*Rev. Inst. Med. trop. S. Paulo*
*45(1):17-21, January-February, 2003*

# MOLECULAR TYPING OF DENGUE VIRUS TYPE 2 IN BRAZIL

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## SUMMARY

Strain typing is a critical tool for molecular epidemiological analysis and can provide important information about the spread of dengue viruses. Here, we performed a molecular characterization of DEN-2 viruses isolated in Brazil during 1990-2000 from geographically and temporally distinct areas in order to investigate the genetic distribution of this serotype circulating in the country. Restriction site-specific polymerase chain reaction (RSS)-PCR presented the same pattern for all 52 Brazilian samples, showing the circulation of just one DEN-2 variant. Phylogenetic analysis using progressive pairwise alignments from 240-nucleotide sequences of the E/NS1 junction in 15 isolates showed that they belong to genotype III (Jamaica genotype).

**KEYWORDS**: Dengue virus type 2; Molecular epidemiology; Brazil.

## INTRODUCTION

Dengue (DEN) viruses are grouped as four antigenically distinct serotypes designated DEN-1, 2, 3, and 4 (SABIN, 1952; HAMMON *et al*., 1960). Evidence that intratypic strain variation occurs among DEN viruses has been demonstrated since the 1970s (McCLOUD *et al*., 1971; RUSSEL & McCOWN, 1972), although only with the advances in molecular technologies it has been possible to determine the genetic variability of each serotype.

Sequencing replaced the original fingerprinting assays, since it allowed a greater discrimination of genetic relations among samples. Partial sequencing of DEN viruses characterized five genomic groups for DEN-1 and DEN-2, four for DEN-3 and two for DEN-4 viruses (RICO-HESSE, 1990, LANCIOTTI *et al*., 1994, 1997).

For DEN-2 viruses the five genotypes are represented by samples from Caribbean and South Pacific (genotype I); Taiwan, Philippines, the New Guinea prototype virus and Thailand 1964 strain (genotype II); Vietnam, Jamaica and Thailand strains (genotype III); Indonesia, Seychelles, Burkina Faso and Sri Lanka (genotype IV) and rural Africa (genotype V) (RICO-HESSE, 1990).

Genome sequencing provides accurate characterization of strain differences but requires labor-intensive procedures. Recently, a new PCR-based approach has been used to rapidly subtype dengue viruses. The method consists of a single reverse transcriptase PCR (RT-PCR) amplification using four primers that target regions spanning polymorphic endonuclease restriction site-specific (RSS-PCR) within the envelope (E) gene (HARRIS *et al*., 1999; MIAGOSTOVICH *et al*., 2000). It was validated against well characterized panels of dengue viruses and was shown to correlate with previous classifications determined by sequence analysis, thus functioning as an alternative subtyping method.

A variety of methods have been used to monitor the distribution and movement of distinct dengue virus genotypes within each serotype, and such genotypic analysis has provided important information about dengue viral spread, transmission and severity of disease with certain genotypes (RICO-HESSE *et al*., 1997).

In this study, we analyzed DEN-2 viruses isolated in Brazil between 1990 and 2000 from geographically and temporally distinct areas to investigate the genetic distribution and diversity of this serotype circulating in the country.

## MATERIALS AND METHODS

*Viruses* - The DEN-2 viruses analyzed in this study were obtained from the collection of the Flavivirus Laboratory, Department of Virology, IOC - FIOCRUZ. The number of each strain, in addition to the location and year of isolation, are listed in Table 1. Viruses were isolated from sera by inoculation into *Aedes albopictus* cell line C6/36 (IGARASHI, 1978) and were identified by immunofluorescence using type-specific monoclonal antibodies (GUBLER *et al*., 1984). Viral seeds were propagated once in C6/36 grown in Leibovitz-15 medium containing 10% fetal bovine serum.

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq- 40.0164/98-1), Oswaldo Cruz Foundation, and the Fogarty International Center of the National Institutes of Health (TW-00905).

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**Table1**
Brazilian dengue-2 isolates used in this study. Location is listed first, followed by year of isolation and strain numbers

| Location | Year | Strain |
|---|---|---|
| Rio de Janeiro | 1990 | 39122[a], 39325[a], 40202, 40247 |
| Ceará | 1994 | 48586 |
| Bahia | 1994 | 48643, 48844 |
| Rio de Janeiro | 1995 | 49255, 49299, 49346, 49367, 50019 |
| Bahia | 1995 | 51502[a] , 51504[a] |
| Espírito Santo | 1995 | 52565, 52582[a], 52634 |
| Bahia | 1996 | 55803, 55968[a] |
| Espírito Santo | 1996 | 56310, 56352, 56565, 57113 |
| Rio de Janeiro | 1996 | 56729 |
| Rio Grande do Norte | 1997 | 59382 |
| Ceará | 1998 | 60442 |
| Rio Grande do Norte | 1998 | 60943, 61654[a] , 64020[a] |
| Rio de Janeiro | 1998 | 61321[a] , 62515[a] , 63597, 63731 |
| Rio de Janeiro | 1999 | 64325, 64625, 64627[a], 64853, 64904, 64905[a] |
| Rio de Janeiro | 2000 | 66977, 66985[a], 66994, 67066, 67955, 68175 |
| Espírito Santo | 2000 | 66690, 66691, 66693, 66703[a], 66718[a], 66724, 66728 |

[a] sequenced isolates

*RNA extraction* - Viral RNA was extracted from the supernatant of infected cells using QIAamp Viral RNA Mini Kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's instructions.

*(RSS)-PCR.* - The reaction mixture and electrophoresis conditions were performed as described previously, except that 25 µl reaction volumes were used (HARRIS *et al.*, 1999). Briefly, 2.5 µl of viral RNA was added to 22.5 µl of an RT-PCR mixture consisting of 50 mM potassium chloride, 10 mM Tris (pH 8.5), 0.01% gelatin, 200 µM of each of the four deoxynucleotide triphosphates, 1.5 mM magnesium chloride, 30 mM tetramethylammonium chloride, 0.5 M betaine, 5 mM dithiotreitol, 0.5 µM of each of four specific RSS-PCR primers for DEN-2 (RSS1-4), 0.025 U/µl of reverse transcriptase RAV-2 (Amersham Corp., Arlington Heights, IL) and 0.025 U/µl of Taq DNA polymerase (AmpliTaq®, Perkin Elmer Corp., Foster City, CA). Reverse transcription was conducted at 42 °C for 60 min, followed directly by 30 amplification cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 5 min. Amplification was conducted in 0.5 ml tubes (USA Scientific, Ocala, FL) in a PTC-200 thermocycler (MJ Research, Inc., Watertown, MA). Ten microliters of the product were analyzed by electrophoresis in a 1.5% agarose gel in 1X TBE (89 mM Tris borate, 2 mM EDTA, pH 8.3) containing 4 ng/ml ethidium bromide. A 100-basepair ladder was used as a size standard (Gibco BRL). The products predict by pairs of primers are as follows: RSS3-RSS4 = 848bp; RSS2-RSS3 = 754bp; RSS1-RSS4 = 676bp; RSS1-RSS2 = 582bp.

*RT-PCR amplification* - Nucleotides from positions 2311 to 2550 coding for the fragment of E/NS1 gene junction were amplified using RT-PCR. The primers, reaction mixture and electrophoresis conditions were performed as previously described (RICO-HESSE *et al.*, 1997). Briefly, 5 µl of viral RNA were added to Superscript II RT buffer (Gibco BRL), 100 mM DTT and 50 pmoles antisense primer D2/2578. The solution was heated at 90 °C for 90 sec and cooled down on ice. Each of the four deoxynucleotides was added (1mM) along with 40 U RNasin (Promega) and 200 U reverse transcriptase Superscript II RT (Gibco BRL). The mixture was incubated for 1 h at 42 °C and cooled on ice. Both PCR primers (150 pM), PCR buffer consisting of 100 mM Tris pH 8.5, 500 mM KCl, 15mM $MgCl_2$ and 0.1% gelatin, and 200 µM of each deoxynucleotide were added to the mix, and the volume was brought up to 50 µl with water. Taq polymerase (5U/Gibco BRL) was added last, and the solution was overlayed with mineral oil. The samples were placed in a thermal cycler (Perkin Elmer CENS) for 30 cycles of 94 °C for 60 sec, 55 °C for 2 min, and 72 °C for 3 min.

*Sequencing* - The RT-PCR product was precipitated with 3M sodium acetate, pH 5.2 and ethanol, electrophoresed in a 1% agarose gel and stained with 1 µg/ml ethidium bromide. Bands (408 bp) were excised and purified using the QIAquick Gel extraction Kit (Gibco BRL). Purified DNA was then sequenced using Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystem, Inc, USA). Cycle sequencing parameters used were as described by the manufacturer's protocol. Sequencing was performed using an ABI Prism 310 (Applied Biosystem). Dendrogram were generated by progressive pairwise alignment (Genetics Computer Group, INC. Wisconsin Package version 9.1).

## RESULTS

The analysis of RSS-PCR products from 52 isolates of Brazilian DEN-2 viruses showed that all of them presented the same pattern, except in a few cases, where extra bands were observed (Fig. 1A, lanes 12-16; Fig. 1B). Although Brazilian RSS-PCR pattern was consistent, it did not match any of the DEN-2 RSS-PCR patterns previously described by HARRIS *et al.*, 1999 (Fig. 1A, lanes 2-8). The Brazilian pattern presented consistent and reproducible bands of 582bp and 100bp and, occasionally extra bands of 676 bp or 150 bp.

Fig. 2 shows a dendogram generated by pairwise alignment of the nucleotide sequence by using progressive pairwise alignments of 15 DEN-2 Brazilian isolates and 24 DEN-2 isolates obtained from GenBank, representing the 5 genotypes described by RICO-HESSE, 1990. According to the dendogram generated all Brazilian DEN-2 viruses are placed together in the same group of Jamaica 81 (GenBank Access # M32950) strain showing that Brazilian strain was more closely related to Asian-like DEN-2 American strain.

The comparison of the 240-base pair sequence spanning the gene junction between the E structural protein and the NS1 nonstructural protein among 15 Brazilian DEN-2 viruses showed homology ranging from 90% to 100% and 94% to 100% for nucleotide sequence and deduced amino acid sequence, respectively. All the nucleotides differences were single base substitutions; no deletions or insertions were observed. In relation to DEN-2 virus isolated from Jamaica 1981, the homology of Brazilian isolates ranged from 95% to 99% for nucleotide and deduced amino acid sequences (Fig. 3).

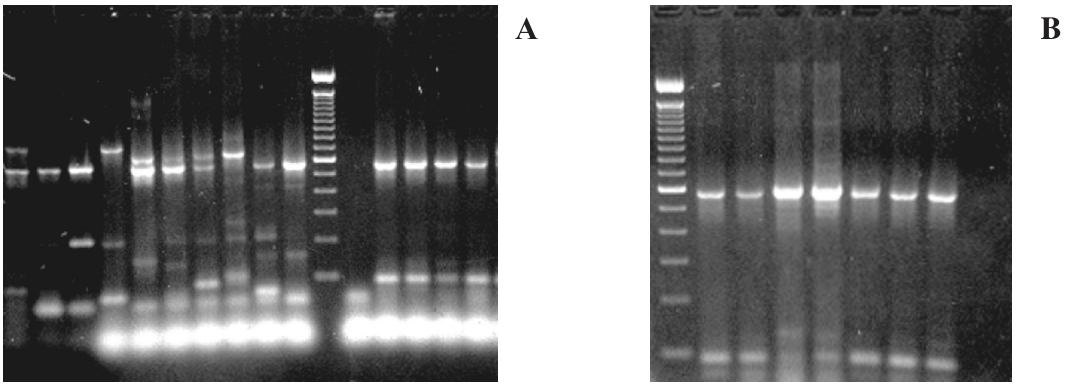


**Fig 1** - Restriction site specific- polymerase chain reaction of dengue-2 viruses. A: agarose gel eletrophoresis of RSS-PCR products from reference isolates and Brazilian DEN-2 isolates; Lane 1: subtype A Puerto Rico strain (202), lane 2 subtype B1: Thailand 1980, strain 218; lane 3: subtype B2: Thailand 1980, strain 603; lane 4: subtype C: Trinidad 1978, strain 144; lane 5: subtype D: Philippines 1984, strain 215; lane 6: subtype E1: Philippines 1975, strain S-35179; lane 7: subtype E2: Sri Lanka 1982, strain 180; lane 8: subtype E3: Indonesia 1977, strain 1174/480; lane 9: subtype F1: Puerto Rico 1977, strain 489; lane 10: subtype F2: Puerto Rico, strain 622, lane 11: molecular weight 100 bp ladder (Gibco BRL); lane 12: $H_2O$, lane 13: Ceará 1994, strain 48586; lane 14: Ceará 1998, strain 60442; lane 15: Bahia 1994, strain 48844; lane 16: Bahia 1995. B: agarose gel eletrophoresis of RSS-PCR products of Brazilian DEN-2 isolates. Lane 1: Molecular weight 100 bp ladder (Gibco BRL); lane 2: Rio de Janeiro 1990, strain 39122; lane 3: Rio de Janeiro 1995, strain 49255; lane 4: Rio de Janeiro 1998, strain 63597; lane 5: Rio de Janeiro 1998, strain 63731; lane 6: Rio de Janeiro 1999, 64325; lane 7: Espírito Santo 1995, strain 52582 and lane 8: Espírito Santo 1996, strain 56565.

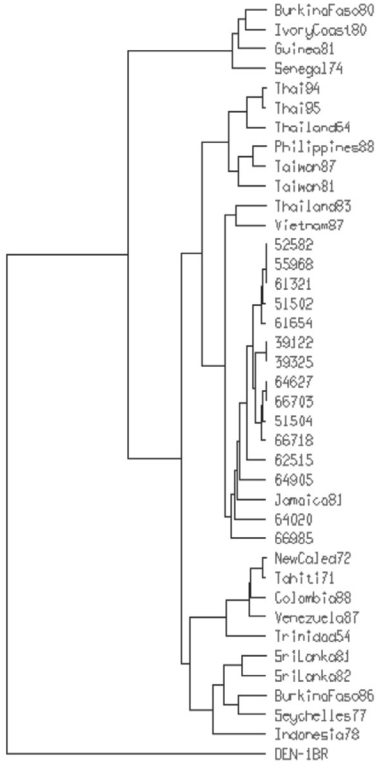


**Fig 2** - Dendogram generated by pairwise alignment of the nucleotide sequence from the E/NS1 junction of 15 DEN-2 Brazilian viruses and 24 obtained from GenBank (Accession Number M32970 (Burkina Faso/1980); M32958 (Ivory Coast/1980); M32966 (Guinea 1981); M32957 (Senegal 1974); U87349 (Thailand 1994); U87382 (Thailand 1995); M32932 (Philippines 1988); M32949 (Taiwan 1987); M32964 (Taiwan 1981); M32947 (Thailand 1983); M32948 (Vietnam 1987); M32950 (Jamaica 1981); M32961 (New Caledonia 1972); M32943 (Tahiti 1971); M32946 (Colombia 1988); M32971(Venezuela 1987); M32969 (Trinidad 1954); M32938 (Sri Lanka 1981); M32940 (Sri Lanka 1982); M32956 (Burkina Faso 1986); M32952 (Seychelles 1977); M32934 (Indonesia 1978); M32923 (DEN-1 BR).

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          1                                                  50
39122 RJ90 .......... .......... .......... .......... ..........
39325 RJ90 .......... .......... .......... .......... ..........
51502 BA95 T......... .......... .......... .......... ..........
51504 BA95 .......... .......... .......... .......... ..........
52582 ES95 .......... .......... .......... .......... ..........
55968 BA96 .......... .......... .......... .......... ..........
61321 RJ98 .......... .......... .......... .......... ..........
61654 RN99 .......... .......... .......... .......... ..........
62515 RJ98 .......... .......... .......... ...IM..... ..........
64020 RN98 ....V..... ...G...... ......G... .......... ..........
64627 RJ99 .......... .......... .......... .......... ..........
64905 RJ99 .......... .......... .......... ..T....... ......DR..
66703 ES00 .......... .......... .......... .......... ..........
66718 ES00 .......... ........E. .......... .......... ..........
66985 RJ00 .......... H......... .......... .......... .....DH...
 Jamaica81 IGVIITWIGM NSRSTSLSVS LVLVGVVTLY LGAMVQADSG CVVSWKNKEL

          51                          80
39122 RJ90 .......... .......... ..........
39325 RJ90 .......... .......... ..........
51502 BA95 .......... .......... ..........
51504 BA95 .......... .......... ..........
52582 ES95 .......... .......... ..........
55968 BA96 .......... .......... ..........
61321 RJ98 .......... .......... ..........
61654 RN99 .....M.... .......... ..........
62515 RJ98 .....M.... .......... ..........
64020 RN98 .......... .......... ..........
64627 RJ99 .......... .......... ..........
64905 RJ99 .......... ......G... .P........
66703 ES00 .......... .......... ..........
66718 ES00 .......... .......... ..........
66985 RJ00 .......... .......... ..........
 Jamaica81 KCGSGIFITD NVHTWTEQYK FQPESPSKL
```

**Fig 3** - Amino acid sequences deduced from the 240 nucleotide sequence of the E/NS1 junction of 15 Brazilian DEN-2 viruses isolated from geographically and temporally distinct areas.

## DISCUSSION

DEN-2 virus was first isolated in the American continent in 1953 in Trinidad (ANDERSON *et al*., 1956) and was responsible for epidemics of classic dengue fever in the 1960s and 70s (GUBLER, 1992). This genotype, called "Puerto Rico", is genetically related to other viruses

from the Pacific Region and India (TRENT *et al*., 1983; RICO-HESSE, 1990) and was the only DEN-2 genotype circulating in the Americas until the 80's, when a new virus related to isolates from Southeast Asia (RICO-HESSE, 1990; LEWIS *et al*., 1993) was introduced during an extensive epidemics in the Caribbean islands in 1981. This genotype, known as "Jamaica", is associated with severe disease and its introduction into the continent resulted in an increase in both numbers of severe cases and epidemics in which there was a significant number of dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) cases (RICO-HESSE *et al*., 1997). For instance, circulation of this variant in 1994-96, especially in Venezuela, resulted in a large number of DHF/DSS cases and high cases fatality (SALAS *et al*., 1998). The "Jamaica" genotype is closely related to the Southeast Asian genotype; in fact recent analysis have merged the two genotypes into one (RICO HESSE *et al*., 1998).

In Brazil, DEN-2 virus was first isolated in 1990 in the State of Rio de Janeiro during an effective dengue surveillance program that included the ongoing systematic collection and sample analysis of suspected cases (NOGUEIRA *et al*., 1990). The introduction of DEN-2 virus increased the number of severe disease cases with the appearance of several cases of DHF/DSS (NOGUEIRA *et al*., 1993; ZAGNE *et al*., 1994). Beginning with its introduction in 1990 until 2000, a total of 928 DHF/DSS cases were reported with 48 deaths (MINISTRY OF HEALTH, BRAZIL).

The DEN-2 virus genetic analysis performed in the present study confirms previously data obtained by using different protocols that identified the "Jamaica" genotype as the only one circulating in the country (RICO-HESSE, 1990; DEUBEL *et al*., 1993; VORNDAM *et al*., 1994; MIAGOSTOVICH *et al*., 1998).

By using a low cost subtyping method (RSS-PCR) we were able to analyze larger number of viruses, performing a retrospective study and characterizing recent isolates during an epidemic period. Although we could not match the RSS-PCR products obtained with the others previously described by HARRIS *et al.,* 1999, we established a consistent and reproducible pattern for DEN-2 Brazilian isolates. The RSS-PCR method was developed with DEN-2 isolates obtained from 1964-1986 and the ongoing evolution of dengue virus over the last 15 years could explain the genetic diversity observed in DEN-2 viruses analyzed in this study, since they were isolated between 1990 and 2000.

The nucleotide changes observed by E/NS1 sequencing of these isolates showed the variability produced during ten years of DEN-2 transmission. Despite this variability, all samples belong to the "Jamaica" genotype. Since sequencing of the entire envelope (E) gene in DEN-2 virus (LEWIS *et al*., 1993) established essentially the same genotypic groups as those suggested by E/NS1 sequencing (RICO-HESSE, 1990), we recommend sequencing of this small region in programs of virological surveillance performed in countries with high dengue viruses activity.

For RSS-PCR results our data suggested that further studies should be performed using different geographic and temporally samples for a better representation of those genotypes since the method showed to be reproducible and can be used as an important tool for assessing the genetic variability of larger numbers of samples.

## RESUMO

### Tipificação molecular do vírus da dengue tipo 2 no Brasil

A tipagem de cepas é uma ferramenta fundamental para a análise epidemiológica molecular e pode fornecer informações importantes sobre a dispersão dos vírus dengue. Neste trabalho, foi realizada a caracterização molecular de amostras de vírus DEN-2 isoladas no Brasil entre 1990-2000, de áreas geograficamente e temporalmente distintas, com o objetivo de investigar a distribuição genética deste sorotipo circulante no país. A reação em cadeia pela polimerase baseada em sítios de restrição específicos (RSS-PCR) apresentou o mesmo padrão para as 52 amostras Brasileiras, mostrando a circulação de apenas uma variante de vírus DEN-2. A análise filogenética utilizando alinhamento progressivo de sequências de 240 nucleotídeos da junção E/NS1 de 15 cepas mostrou que estas pertencem ao genotipo III (genotipo Jamaica).

## ACKNOWLEDGMENTS

To José da Costa Farias Filho and José de Carvalho Filho for dengue virus isolation and identification and to the Directors of Central Laboratories from Bahia, Ceará, Espírito Santo and Rio Grande do Norte for supplying serum samples.

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Received: 24 July 2002
Accepted: 22 October 2002